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Persistence of Antifolate Activity in Skin of Rats Following Systemic Administration of Methotrexate

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Studies of the time course of methotrexate-derived antifolate activity (methotrexate activity) in plasma and skin of rats have been carried out to evaluate the hypothesis that the formation of therapeutically active poly-γ-glutamyl conjugates of methotrexate in skin after systemic administration of the drug will result in the prolongation of methotrexate effects in skin. Such a finding may eventually bear on the use of the drug in the treatment of psoriasis. Three groups of 4 rats received a single i.v. dose of methotrexate, 5, 20, or 40 mg/kg, respectively. Serial skin and plasma samples were obtained over 48 h from each rat and analyzed for methotrexate activity by a dihydrofolate reductase enzyme inhibition assay. The values of pharmacokinetic parameters describing the disposition of the drug were calculated from plasma data using standard methods. Skin to plasma ratio of methotrexate activity was also calculated each time a skin sample was obtained. Pharmacokinetic parameter values and skin to plasma ratios were not significantly different between doses. Methotrexate activity declined biexponentially in plasma and skin. The terminal half-life in plasma (mean ± SD) was 20.3 ± 9.2 h. At early times, methotrexate activity in skin was less than plasma, but at later times activity in skin was an order of magnitude greater than that in plasma. In a preliminary study to determine whether the persistent activity in skin is associated with the presence of poly-γ-glutamyl conjugates of methotrexate, a rat was dosed with tritiated methotrexate, 0.18 mg/kg, i.v., and skin was obtained 24 h later. Skin homogenate was treated with papain and applied to an anion-exchange column for cleanup prior to injection on a reversed-phase high-pressure liquid chromatography system. Methotrexate poly-γ-glutamates were identified by elution with authentic standards and specific hydrolysis with carboxypeptidase G_1.

For more than 20 years, methotrexate (2,4-diamino-N^{10}-methyl-pteroyl-glutamic acid) has been used in the management of severe psoriasis [1] and is considered to be one of the most useful drugs in patients in whom the disease has resisted all other forms of therapy [2]. Since a larger than normal proportion of psoriatic cells are in the S phase (DNA-synthesizing phase) of the cell cycle, psoriatic tissue [3] and the individual psoriatic cells [4] are very susceptible to S phase-specific agents such as methotrexate [5,6].

Methotrexate is given orally or parenterally for the treatment of psoriasis. The most common dosage regimen is 3 or 4 2.5–5.0 mg oral doses during a period of 32 or 36 h on a weekly basis [7]. This regimen is based on the 36-h duration of the S phase of psoriatic cells and the duration of action of methotrexate following a single dose [4,8]. This regimen appears to result in less long-term hepatic toxicity than is observed with other dosage regimens [9–11].

Work describing the formation of polar active metabolites of methotrexate, the poly-γ-glutamyl conjugates, in cultured human skin fibroblasts has been carried out since the regimen of weekly exposure to methotrexate was adopted [12]. Since these conjugates are retained in cells, subsequent doses of methotrexate may have much longer duration of action than the first. An understanding of the kinetics of accumulation of these conjugates could therefore be important in further refining the regimen for administration of methotrexate for the treatment of psoriasis. The present study was undertaken to delineate the time course of antifolate activity in the skin and plasma of rats following the systemic administration of methotrexate, and to determine whether methotrexate poly-γ-glutamates are formed in the skin.

MATERIALS AND METHODS

Animal Procedures

Male Sprague-Dawley rats (250–370 g) received jugular cannulas under light ether anesthesia. Atropine sulfate, 320 μg/kg was administered i.p. to inhibit salivation during the surgical procedure. Hair was removed from the abdomen of the rat with animal clippers at the time of surgery. After surgery, the rats were rested for 1 day and received food and water ad libitum. The cannula was kept patent by flushing approximately every 8 h with heparinized normal saline (0.2 ml of a 10 U/ml solution). On the day after surgery, the rats were anesthetized with ether, and a dose (5, 20, or 40 mg/kg) of methotrexate (Lederle) was injected into a tail vein. These doses were selected to allow determination of antifolate activity in serially obtained plasma and skin samples from individual animals. A group of 4 rats received each dose. Blood was sampled through the jugular cannula at 0.17, 0.5, 1, 2, 4.5, 8, 14, 22, 30, and 48 h and centrifuged in heparinized capillary tubes (Caraway Capillary Tubes, Heparinized, American Hospital Supply Corporation). Sample size varied from 0.1 ml at early times to 1.0 ml at late times. Plasma was removed and frozen until analyzed. Skin samples of approximately 40 mg were taken under ether anesthesia from the shaved abdomen at 0.25, 0.75, 4, and 14 h, blotted, weighed, and frozen immediately. The biopsy site was sutured closed. At 48 h after the dose, the rat was again anesthetized, exsanguinated through the abdominal aorta, and a final skin sample of approximately 150 mg was obtained.

Determination of Methotrexate Activity

Skin was processed according to the method of Bertino and Fischer [13] with slight modifications. Samples were thawed, minced, and diluted with 10 volumes of 0.5 M Tris, pH 7.5. They were then homogenized using a drill-driven Teflon pestle inside a glass tube immersed in an ice bath. The homogenate was immersed in boiling water for 10 min before centrifugation at 2000 *g* for 15 min. The supernatant was recovered and analyzed for methotrexate-derived antifolate activity (methotrexate activity).

Methotrexate activity in skin and plasma was determined by an enzyme inhibition assay based upon that by Falk et al [14]. Dihydrofolic acid is converted to tetrahydrofolic acid by dihydrofolate reductase in the presence of NADPH. Methotrexate competitively inhibits this reaction, and its concentration can be monitored by comparing the

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Abbreviations:
ANOVA: one-way analysis of variance
HPLC: high-pressure liquid chromatograph(y)
MTX: methotrexate

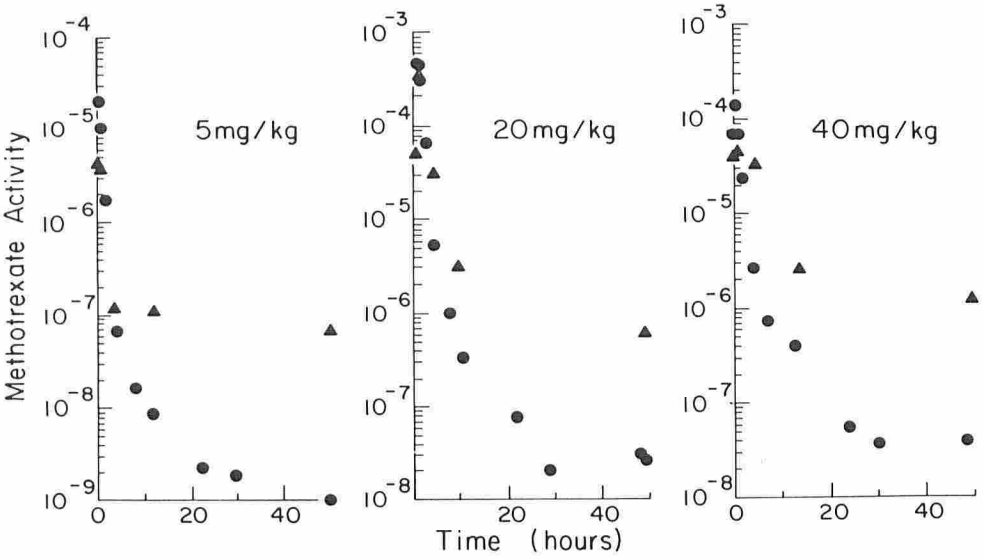

FIG 1. Representative plots of the time course of methotrexate activity expressed as mole/L (M) in plasma (●) and mole/kg in skin (▲) of rats following methotrexate doses of 5, 20, or 40 mg/kg, i.v.

change in the concentration of NADPH determined by absorbance at 340 nm in the sample to the change in absorbance in an uninhibited control. The cuvette compartment of a DW-2 spectrophotometer (American Instrument Company) was maintained at 37°C with a circulating water bath. An aliquot of the standards or sample was placed in a 1-ml cuvette containing NADPH (Sigma), dihydrofolate reductase from *Lactobacillus casei* (New England Enzyme Center) and potassium chloride (Sigma) dissolved in the Tris buffer (Trizma base, Sigma). This mixture was inverted several times by hand and allowed to incubate for 3 min in the cuvette compartment. Dihydrofolic acid (Sigma) was then added, the cuvette was again inverted several times, and absorbance was recorded on the X-Y plotter for 6 min. Using the linear portion of the absorbance curve thus generated, the absorbance change in 5 min for a standard or sample containing methotrexate was subtracted from the absorbance change in 5 min for the mixture lacking methotrexate. A linear calibration curve was constructed using concentrations of methrotrexate ranging from 2×10^{-10} M to 2×10^{-9} M. Thus, methotrexate-derived antifolate activity is expressed in units of methotrexate concentration. Because the reaction was not linear outside this range, plasma samples and skin supernatants were diluted into this range with water when necessary. The difference in the concentration of a given standard determined at the beginning and end of a day was, on an average, less than 1%, with a coefficient of variation of 16%. Day-to-day variation was measured by running a rat plasma control containing 1×10^{-8} M methotrexate each time the assay was run. The concentrations in these controls ranged from 6.88×10^{-9} M to 1.13×10^{-8} M with an average of 9.40×10^{-9} M and an SD of 1.26×10^{-9} M (coefficient of variation 13.4%). These results compare well with previous reports using this technique. The lower limit of detection in plasma and skin supernatant was 1×10^{-10} M.

Poly-γ-glutamate Conjugates

[3′,5′,7-^{3}H]Methotrexate (Amersham) was purified on a Sephadex G-15 column (Bio-Rad, 0.9 × 55 cm) which had been equilibrated and washed with a buffer consisting of 25 mM sodium phosphate, 100 mM NaCl, and 0.02% NaN_3, pH 7.0 as described by Whitehead et al [15]. The final specific activity was 0.644 μCi/μg, and 180 μg/kg was injected into the tail vein of a 396-g male Sprague-Dawley rat. Hair was removed from the abdomen with clippers. Twenty-four hours later, the rat was anesthetized with ether and 6.6 g of skin was obtained. The procedure used to isolate poly-γ-glutamyl conjugates of methotrexate is based on that reported by Hoppner and Lampi [16] for folic acid in fortified foods.

Skin was immediately minced with scissors, diluted 6:1 with hot buffer (phosphate/citrate, 0.05 M, pH 8.0, with 8.5 mM ascorbic acid and 0.1 mM $ZnCl_2$) and placed in a boiling water bath for 10 min. Boiling was followed by homogenization (Thomas hand homogenizer, Teflon pestle driven by an electric drill, glass vessel) for 3 passes. The sample was again placed in a boiling water bath for 5 min, then cooled to room temperature, and placed in a 40°C water bath. Papain (50 mg) (Sigma) in 1 ml diluent (H_2O:0.01 M EDTA:0.06 M mercaptoethanol:0.05 M cysteine, 70:10:0.1:10) [17] was added and the mixture was incubated for 12 h. Preliminary studies showed that this step did not degrade methotrexate or its poly-γ-glutamate conjugates. The supernatant was removed, filtered through a Whatman GF/A glass fiber filter disc (4.25 cm), and the residue was washed with 5 ml phosphate/citrate buffer, 0.005 M, pH 8.0. A portion of the filtrate was placed on a DEAE/cellulose column (Cellex D, Bio-Rad, equilibrated with 0.005 M phosphate buffer, pH 7.0) and eluted with 10 ml 0.05 M phosphate buffer (pH 7.0) followed by a second buffer consisting of 0.1 M phosphate + 0.5 M NaCl (pH 7.0). The second portion of the filtrate was incubated overnight with carboxypeptidase G_1 (New England Enzyme Center) at 37°C prior to DEAE-cellulose chromatography. Forty fractions of approximately 4.35 ml were collected in each case. The amount of radioactivity was determined in each fraction by liquid scintillation counting. Fractions comprising individual peaks were combined and an aliquot was injected on the high-pressure liquid chromatograph (HPLC) together with authentic standards of methotrexate, MTX-G_2, MTX-G_3, and MTX-G_7*. Metabolites were obtained from Dr. Charles M. Baugh, University of South Alabama [18]. To aid in identification of hydrolysis products, authentic standards were also incubated with carboxypeptidase G_1 and injected onto the HPLC.

The HPLC system included a 10-μm C_{18} μBondapak column (Waters) maintained at 50°C. The mobile phase was 11% acetonitrile in 0.6 M glycine/HCL buffer, pH 3.45, delivered at a rate of 1 ml/min. Eluate was routed through a 254 nm UV detector (Waters 440) prior to collection of fractions. One-minute fractions were collected in scintillation vials, and 10 ml Aquasol-2 (New England Nuclear) was added prior to counting. The recovery of radioactivity through the entire procedure is 89 ± 9%.

Pharmacokinetic Calculations

Pharmacokinetic parameters were calculated for each animal by standard methods [19]. Plasma clearance (Cl) was calculated as the

TABLE I. *Effect of dose on the pharmacokinetics of methotrexate in the plasma of rats*

Dose (mg/kg)	Clearance (ml/min/kg)	Volume of distribution (ml/kg)[a]	Half-life (hr)
5	10.8 ± 5.7[b]	932 ± 497	15.1 ± 5.4
20	7.6 ± 9.1	756 ± 907	27.6 ± 8.9
40	7.8 ± 2.2	859 ± 230	18.1 ± 9.5
	N.S.[c]	N.S.	N.S.
Overall	8.8 ± 5.9[d]	849 ± 558	20.3 ± 9.2

[a] Volume of distribution at steady-state.
[b] Mean ± SD, n = 4.
[c] No significant difference between doses by ANOVA.
[d] Mean ± SD, n = 12.

* The subscript associated with G refers to the total number of glutamic acid residues contained in the molecule, and not the number of residues added to methotrexate. Methotrexate itself contains a single glutamic acid residue.

ratio of dose to the area under the plasma activity-time curve (AUC). Half-life was calculated as 0.693 divided by the absolute value of the terminal slope (determined by linear least squares regression) of the semilogarithmic plot of activity vs time. The volume of distribution at steady-state (*Vss*) was calculated as

$$Vss = \frac{Dose \cdot AUMC}{(AUC)^2}$$

where AUMC is the area under the first moment of the plasma activity-time curve. Since in most cases the plasma samples were not taken at exactly the same time as the skin samples, plasma activities were logarithmically interpolated to give an estimate of plasma activity at the time of skin sampling for the determination of skin/plasma activity ratio.

The means and SD of the pharmacokinetic parameters and the skin/plasma ratios were calculated within each dosing group. The pharmacokinetic parameters were then compared to one another by means of one-way analysis of variance (ANOVA) in order to determine whether any parameter was dose-dependent [20]. Additionally, the means and SD of the pharmacokinetic parameters and the skin/plasma ratios of methotrexate activity were calculated for all 12 animals as a group. Growth curve analysis was performed on the skin/plasma activity ratios to determine whether the ratios were dose and/or time-dependent [21].

TABLE II. *Skin/plasma ratio of methotrexate activity as a function of time and dose*

Dose (mg/kg)	Time (hours)				
	0.25	0.75	4.0	14.0	48.0
5	1.21 ± 1.94[a]	0.73 ± 0.46	1.25 ± 0.63	36.1 ± 27.7	N.D.[b]
20	0.81 ± 1.31	0.74 ± 0.46	1.14 ± 0.33	18.2 ± 13.9	9.59 ± 9.20
40	0.32 ± 0.15	0.85 ± 0.65	2.83 ± 3.80	9.21 ± 3.26	22.2 ± 20.3
	N.S.[c]	N.S.	N.S.	N.S.	N.S.
Overall[d]	0.78 ± 1.28	0.78 ± 0.48	1.74 ± 2.17	21.2 ± 20.0	15.9 ± 16.3

[a] Mean ± SD, n = 4.
[b] Not determined, plasma activity below lower limits of detection.
[c] No significant difference between doses, growth curve analysis [20].
[d] Mean ± SD, n = 12 (n = 8 at 48 h). Differences between times significant ($p < 0.05$), growth curve analysis.

RESULTS

The decline of methotrexate activity in plasma after single i.v. doses of either 5, 20, or 40 mg/kg was biexponential, consisting of an initial rapid decrease followed by a slower log-linear phase (Fig 1). Methotrexate activity in skin was less than that in plasma at early times; thereafter activity in skin was much greater than in plasma. Because few determinations of methotrexate activity in skin were made in the terminal log-linear phase, it was not appropriate to calculate a half-life in the skin. However, the decline of methotrexate activity in the skin was qualitatively similar to that in plasma.

There was no difference in the plasma pharmacokinetic parameters between the 3 different dosing groups as measured by one-way ANOVA (Table I). The 12 rats were therefore treated as a single group and the overall values of plasma pharmacokinetic parameters were calculated. Pharmacokinetic parameter values could be calculated because methotrexate activity in plasma was due solely to the drug itself (unpublished observation). Plasma clearance of methotrexate was 8.8 ± 5.9 ml/min/kg (mean ± SD), the steady-state volume of distribu-

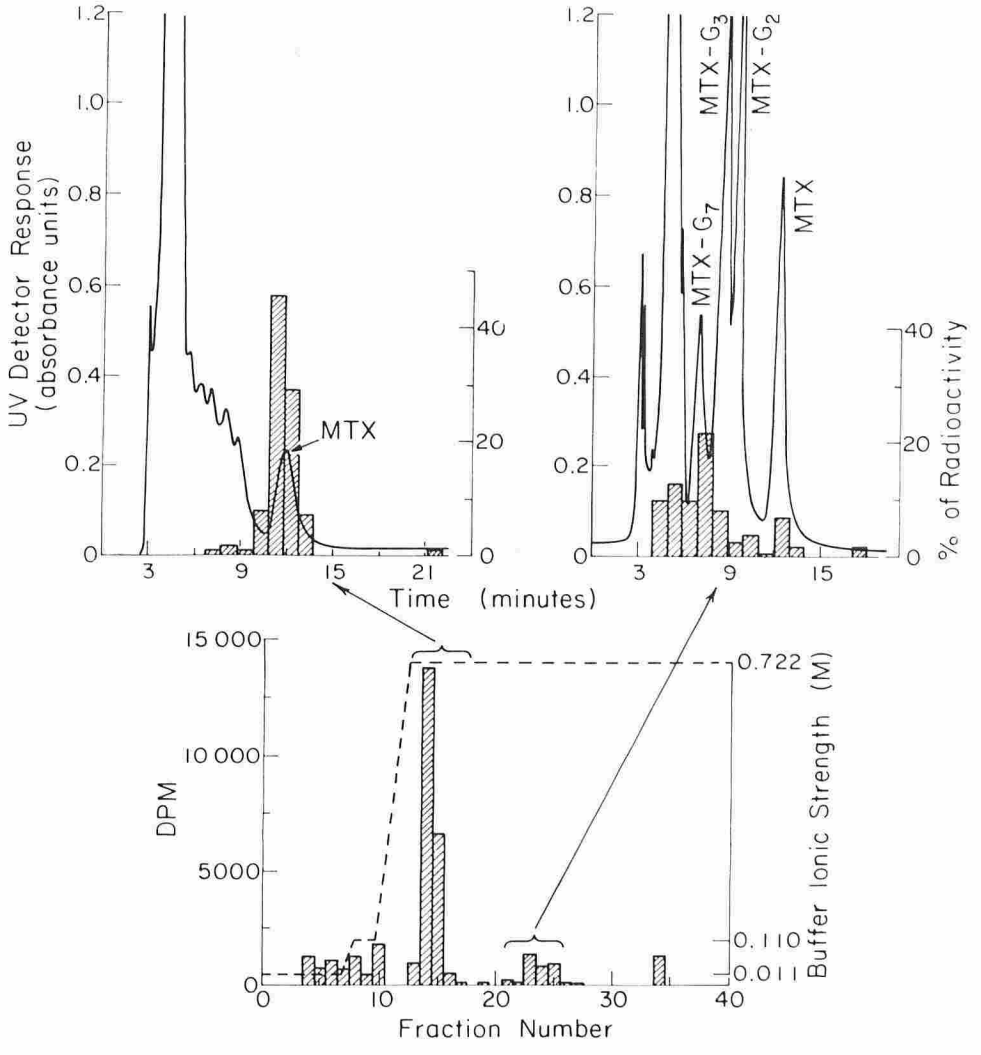

FIG 2. Composite of DEAE-cellulose chromatography *(bottom)* followed by reversed-phase HPLC analysis *(top)* of a skin sample obtained from a rat that received [^{3}H]methotrexate. The *hatched bars* in the lower portion of the figure represent the radioactivity detected in fractions eluted from the DEAE-cellulose anion-exchange column as the ionic strength of the buffer was increased (stippled line). An aliquot of fractions 13–17 was injected on the HPLC column with cold carriers; fractions were collected and radioactivity detected as shown by the *upper left* portion of the figure. An aliquot of fractions 21–27 was injected on the HPLC column with cold carriers; fractions collected and radioactivity detected as shown by the *upper right* portion of the figure. The *solid line* in the HPLC chromatographs is the UV detector trace of authentic cold carrier standards, *hatched bars* represent radioactivity from the skin sample. (MTX, methotrexate; MTX-G_2, methotrexate mono-γ-glutamate; MTX-G_3, methotrexate di-γ-glutamate; MTX-G_7, methotrexate hexa-γ-glutamate.)

tion was 849 ± 558 ml/kg and plasma half-life was 20.3 ± 9.2 h.

Skin/plasma activity ratios (Table II) were examined by growth curve analysis for dose and time dependence. Since 3 of 4 rats receiving a 5 mg/kg dose had no detectable methotrexate activity in the plasma at 48 h, the skin/plasma ratio was not calculated for any of the 4 rats, and they were not included in the analysis. There was no significant difference in the skin/plasma concentration ratio between the 3 different dosing groups at any time point. The analysis did show that there were significant ($p < 0.05$) differences between the overall means as a function of time.

It has been shown that the poly-γ-glutamyl conjugates of methotrexate are formed in human red blood cells [22], human liver [23,24], and rat liver [15,22] and that the half-life of these conjugates in the rat liver is about 6.6 days [15]. Also, these metabolites may be as effective as methotrexate in the inhibition of dihydrofolate reductase [25]. If so, the enzyme inhibition assay described above would not distinguish methotrexate from these metabolites. Additional studies were therefore required to identify the material present in skin.

Fig 2 is a composite of the DEAE-cellulose and reversed-phase HPLC analysis of skin samples from a rat that received [^{3}H]methotrexate. Anion-exchange chromatography (DEAE-cellulose) revealed several peaks of radioactivity in the samples of skin obtained 24 h after the dose. Preliminary studies with authentic standards showed that methotrexate and its poly-γ-glutamates eluted in fractions 13–27. When an aliquot of the peak comprised of fractions 13–17 was injected on the HPLC, a small amount of the poly-γ-glutamyl conjugates of methotrexate was seen by comparing the radioactive content of the eluate with the simultaneous UV detector trace of cold carrier standards. However, HPLC analysis of the peak comprised of fractions 21–27 from the anion-exchange column does strongly suggest the presence of the poly-γ-glutamyl conjugates of methotrexate.

Fig 3 is analogous to Fig 2, except that the papain-digested skin was incubated with carboxypeptidase G_1 (to hydrolyze methotrexate and the glutamate conjugates) prior to chromatography. A single major peak of radioactivity was observed. When an aliquot was injected on the HPLC, a single peak with a retention time of approximately 25 min was observed. A peak with similar retention time is the only one present on HPLC analysis when authentic standards of methotrexate, MTX-G_2, MTX-G_3, and MTX-G_7 are subjected to hydrolysis by carboxypeptidase G_1 prior to HPLC analysis (Fig 4).

The peaks eluting at approximately 25 min in Figs 3 and 4 most likely arise from DAMPA, which is methotrexate minus glutamic acid (2,4-diamino-N^{10}-methylpteroic acid) [26]. The HPLC results shown in Figs 2 and 3 suggest that poly-γ-glutamate conjugates greater than MTX-G_7 are formed in skin because radioactivity eluting before MTX-G_7 in Fig 2 is not present following treatment with carboxypeptidase G_1.

DISCUSSION

Over the range of doses studied, no dose dependence of the pharmacokinetics of methotrexate activity was observed (Table I). Dose-dependence could have been masked by the large degree of interanimal variability in the values of pharmacokinetic parameters. Coefficients of variation for clearance, volume of distribution, and half-life were 68%, 66%, and 45%, respectively. Dose dependence might have been detected with more frequent blood sampling, with a larger number of rats, or might be observed outside the 8-fold range of doses examined.

Of particular relevance to the role of methotrexate in the treatment of psoriasis is the finding of time-dependence, but not dose dependence, in the skin/plasma methotrexate activity ratio (Table II). At late times, methotrexate activity in skin was much greater than that in plasma. A similar observation has been made in patients [27]. In this report, patients who had been receiving the drug for the treatment of malignancies

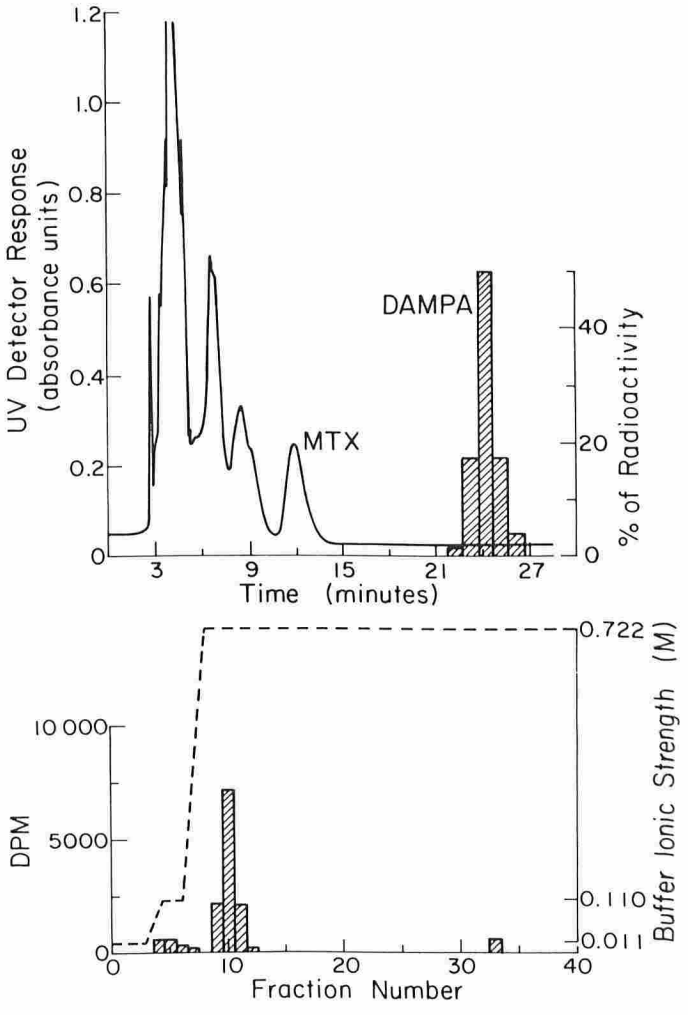

FIG 3. As Fig 2 except that the papain-digested skin homogenate was incubated with carboxypeptidase G_1 before being placed on the anion exchange column. An aliquot of fractions 9–12 was injected onto the HPLC (DAMPA, 2,4-diamino-N^{10}-methylpteroic acid).

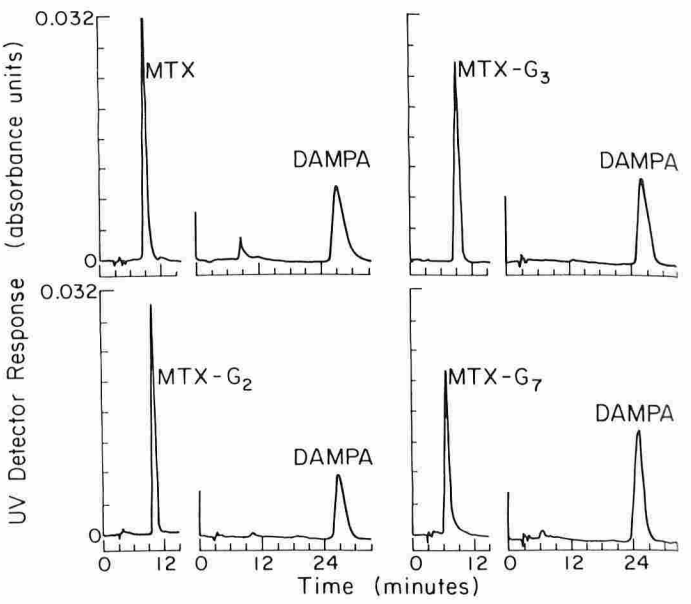

FIG 4. HPLC chromatographs of authentic standards of methotrexate and 3 poly-γ-glutamate conjugates [17]. On the *left* in each panel is the chromatograph of the standard, and on the *right* is the chromatograph of the standard following incubation with carboxypeptidase G_1.

were given a dose of [^{3}H]methotrexate intravenously. The persistence of tritium in skin of patients was very similar to the persistence of antifolate activity in skin of rats observed here. It appears that methotrexate is sequestered in the skin, released slowly to the blood, and then eliminated. Since it has been shown that methotrexate mono-γ-glutamate is equipotent to methotrexate in terms of inhibition of dihydrofolate reductase [25], the assay based upon this enzyme is not specific for methotrexate. Therefore, the prolonged activity in the skin might be due to the poly-γ-glutamyl conjugates and not methotrexate itself.

Additional evidence for this hypothesis was given by work done in cultured human skin fibroblasts. The poly-γ-glutamyl conjugates of methotrexate were formed within the cells when the drug was placed in the culture medium [12]. These compounds were associated with prolonged inhibition of deoxyuridine incorporation into DNA when the cells were transferred into methotrexate-free medium [28]. In studies of cultures of other cell lines, the poly-γ-glutamyl conjugates have been shown to be retained within the cells to a greater extent than methotrexate [29,30], possibly because the cells are less permeable to these more polar compounds. These in vitro studies suggest that methotrexate is taken up into the cells and is metabolized to the poly-γ-glutamyl conjugates, which are selectively retained and are responsible for the prolonged antifolate activity. Studies are presently being carried out to quantitatively determine whether the poly-γ-glutamate conjugates of methotrexate are responsible for the prolonged antifolate activity seen in the skin of intact rats. Preliminary studies reported here demonstrate the presence of these metabolites in skin 24 h after the dose. The data also suggest that poly-γ-glutamates of chain length longer than MTX-G_7 are present in the skin 24 h after administration of a 0.18 mg/kg dose of [^{3}H]methotrexate. Methotrexate poly-γ-glutamates of this length have not been reported in any tissue previously.

The formation of long-acting methotrexate conjugates in skin that bind effectively to dihydrofolate reductase [31,32] and share many of the cytotoxic activities of methotrexate [25,28,32] may have implications for the therapy of psoriasis. In addition, the involvement of an extrahepatic, reversible step in drug metabolism intimately linked to therapeutic and toxic effects is of unique pharmacologic interest.

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